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TECHNICAL REPORT

Development of Enzyme-Containing Functional Nanoparticles

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August 2012

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13. ABSTRACT (Maximum 200 words)

Enzymes demonstrate great potential for numerous applications; however their practicality is limited by instability in harsh environments, such as temperature extremes; limiting both operational stability as well as long-term shelf life. In this work, we seek to overcome enzyme instability at elevated temperatures through the encapsulation of enzymes within poly(*N*-isopropylacrylamide, NiPAAm) colloidal nanoparticles Nanoparticles composed of NiPAAm undergo a transition from being highly hydrophilic and swollen (at room temperature) to shrunken and hydrophobic (at elevated temperatures). We are taking advantage of this phenomenon by encapsulating and covalently immobilizing enzymes within the particles. Enzymes denature at high temperatures, unfolding in the process. Encapsulation within NiPAAm particles prevents denaturation as the shrinking polymer network prevents the enzyme from unfolding at high temperatures.

Glucose oxidase and organophosphorus hydrolase were functionalized with hydrophobic and polymerizable chemical groups and encapsulated within NiPAAm/Styrene particles using an enzyme friendly emulsion polymerization technique. The particles undergo a volume transition from approximately 100nm to 50nm in size.

Thermal stability studies in aqueous conditions were conducted at temperatures up to 70°C, resulting in a 2-10 fold improvement in enzyme pot-life. Lyophilization of these novel materials extends their shelf over that of native enzyme from 3 weeks to over 3 months.

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CONVERSION TABLE

Conversion Factors for U.S. Customary to metric (SI) units of measurement.

MULTIPLY ——→ BY ——→ TO GET

TO GET ←—— BY ←—— DIVIDE

angstrom	1.000 000 x E -10	meters (m)
atmosphere (normal)	1.013 25 x E +2	kilo pascal (kPa)
bar	1.000 000 x E +2	kilo pascal (kPa)
barn	1.000 000 x E -28	meter^2 (m^2)
British thermal unit (thermochemical)	1.054 350 x E +3	joule (J)
calorie (thermochemical)	4.184 000	joule (J)
cal (thermochemical/cm^2)	4.184 000 x E -2	mega joule/m^2 (MJ/m^2)
curie	3.700 000 x E +1	*giga bacquerel (GBq)
degree (angle)	1.745 329 x E -2	radian (rad)
degree Fahrenheit	$t_k = (t°f + 459.67)/1.8$	degree kelvin (K)
electron volt	1.602 19 x E -19	joule (J)
erg	1.000 000 x E -7	joule (J)
erg/second	1.000 000 x E -7	watt (W)
foot	3.048 000 x E -1	meter (m)
foot-pound-force	1.355 818	joule (J)
gallon (U.S. liquid)	3.785 412 x E -3	meter^3 (m^3)
inch	2.540 000 x E -2	meter (m)
jerk	1.000 000 x E +9	joule (J)
joule/kilogram (J/kg) radiation dose absorbed	1.000 000	Gray (Gy)
kilotons	4.183	terajoules
kip (1000 lbf)	4.448 222 x E +3	newton (N)
kip/inch^2 (ksi)	6.894 757 x E +3	kilo pascal (kPa)
ktap	1.000 000 x E +2	newton-second/m^2 ($N-s/m^2$)
micron	1.000 000 x E -6	meter (m)
mil	2.540 000 x E -5	meter (m)
mile (international)	1.609 344 x E +3	meter (m)
ounce	2.834 952 x E -2	kilogram (kg)
pound-force (lbs avoirdupois)	4.448 222	newton (N)
pound-force inch	1.129 848 x E -1	newton-meter (N-m)
pound-force/inch	1.751 268 x E +2	newton/meter (N/m)
pound-force/foot^2	4.788 026 x E -2	kilo pascal (kPa)
pound-force/inch^2 (psi)	6.894 757	kilo pascal (kPa)
pound-mass (lbm avoirdupois)	4.535 924 x E -1	kilogram (kg)
pound-mass-foot^2 (moment of inertia)	4.214 011 x E -2	kilogram-meter^2 ($kg-m^2$)
pound-mass/foot^3	1.601 846 x E +1	kilogram-meter^3 (kg/m^3)
rad (radiation dose absorbed)	1.000 000 x E -2	**Gray (Gy)
roentgen	2.579 760 x E -4	coulomb/kilogram (C/kg)
shake	1.000 000 x E -8	second (s)
slug	1.459 390 x E +1	kilogram (kg)
torr (mm Hg, 0^0 C)	1.333 22 x E -1	kilo pascal (kPa)

*The bacquerel (Bq) is the SI unit of radioactivity; 1 Bq = 1 event/s.

**The Gray (GY) is the SI unit of absorbed radiation.

Project Objective

Enzymes demonstrate great potential for numerous applications; however their practicality is limited by their instability in harsh environments, such as temperature extremes. This limits both operational stability as well as long-term shelf life. In this work, we seek to overcome enzyme instability at elevated temperatures through the encapsulation of enzymes within poly(*N*-isopropylacrylamide, NiPAAm) colloidal nanoparticles which demonstrate a lower critical solution temperature (LCST) shrinkage at temperatures > 32°C. Nanoparticles composed of NiPAAm undergo a transition from being highly hydrophilic and swollen (at room temperature) to shrunken and hydrophobic (at elevated temperatures). We are taking advantage of this phenomenon by encapsulating and covalently immobilizing enzymes within the particles. Enzymes denature at high temperatures, unfolding in the process. Encapsulation within NiPAAm particles prevents denaturation as the shrinking polymer network prevents the enzyme from unfolding at high temperatures.

Glucose oxidase (GOx), a model enzyme, and organophosphorus hydrolase (OPH) were functionalized with hydrophobic and polymerizable chemical groups and encapsulated within NiPAAm/Styrene particles using an enzyme friendly emulsion polymerization technique. The particle contraction at high temperatures, from approximately 100 nm to 50 nm in diameter, enhances enzymatic stability by supporting the enzyme's tertiary structure and preventing unfolding.

Thermal stability studies in aqueous conditions were conducted at temperatures up to 70°C. Catalytic activity of encapsulated enzyme is higher relative to that of native or functionalized enzyme at elevated temperatures, typically resulting in a 2-10 fold improvement in enzyme pot-life. Lyophilization of these novel materials extends their shelf life (dry storage conditions) over that of native enzyme. After 3 weeks of dry storage at 70 °C, native enzyme loses all activity; whereas encapsulated enzyme retains approximately 40% activity after 12 weeks at 70°C. Encapsulation of enzymes extends shelf life from 3 weeks to over 3 months (study is currently ongoing).

We have demonstrated that enzymes retain catalytic activity within NiPAAm nanoparticles. The enzyme is structurally supported via covalent immobilization and is further structurally supported at elevated temperatures by the collapsing NiPAAm network. The ability to maintain or enhance the stability of enzymes at high temperatures could have significant application in the fields of industrial catalysis, decontamination, and field-portable diagnostics.

Tasks for Development of Enzyme-Containing Functional Nanoparticles

Year 2

- ***V. Polymerization of Particles with Modified Enzyme Seeds***
- ***VI. Physical Characterization of Enzyme-Containing Particles***

Year 2 and 3

- ***VII. Core-shell Nanoparticle Response***

Year 3

- ***VIII. Non-Specific Stimuli Testing of Particles***

IX. Assess Continuous Operation Lifetime of Particles

X. Determine Shelf Life of Particles

Accomplishments

Task 5: Polymerization of Particles with Modified Enzyme Seeds

Enzymes are generally inherently hydrophilic on the surface but can be chemically modified with hydrophobic polymerizable groups to make them 'soluble' in organic media. The modification not only allows the enzyme to become more soluble but also to be used a co-monomer in the nanoparticle fabrication procedure, resulting in 3-dimensional covalent attachment to the nanoparticle. Glucose oxidase (GOx) or organophosphorus hydrolase (OPH) were modified in a two step modification procedure by first modifying the enzymes with palmitic acid n-hydroxysuccimide (Palmitic acid NHS). After the first coupling reaction, the enzyme was further modified with acrylic acid n-hydroxysuccimide (aaNHS).

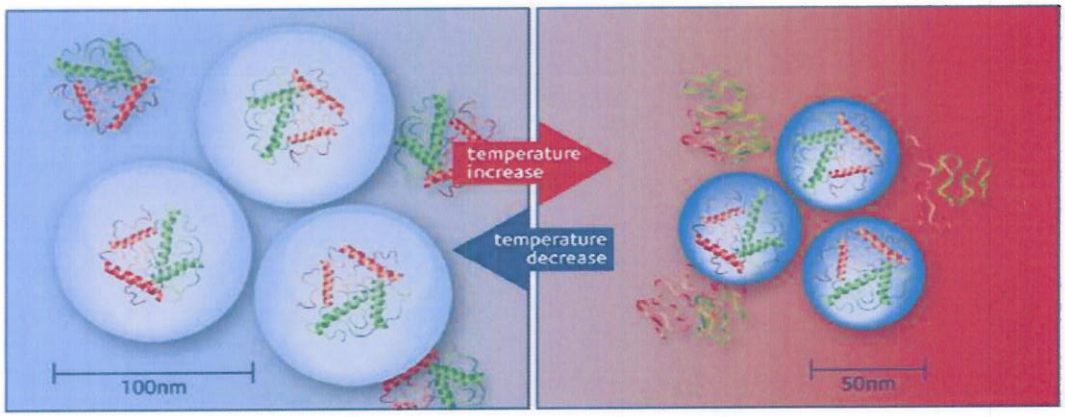

Figure 1. Conceptual representation of the affect temperature has on free enzymes and encapsulated enzyme within *N*-isopropylacrylamide (NiPAAm)/Styrene particles. Free enzymes denature when exposed to increases in temperature, while encapsulated enzyme's tertiary structure is protected by the collapsing particle.

At slightly above ambient temperature conditions, nanoparticles containing modified enzyme seeds were prepared via standard oil-in-water emulsion polymerization protocols. The seed particle (in this case the enzyme-pa-acrylic acid graft) was added to a stirred aqueous solution that contained an anionic surfactant, sodium dihexyl sulfosuccinate (MA-80). MA-80 was added drop wise to deionized water (dH_2O) at 3.5% ($^{v}/_{v}$) which is above its CMC of approximately 1.2 to 1.6% ($^{v}/_{v}$). The concentration of enzyme added to the reactor varied depending upon the amount required for activity assays. Either 50mg of GOx-pa-acrylic acid was added to the 100mL round bottom reactor, for a final concentration of 0.5mg/mL GOx-pa-acrylic acid or 500mg OPH-pa-acrylic acid was added for a final concentration of 5mg/mL OPH-pa-acrylic acid. The functionalized enzyme was added drop wise to the reactor and equilibrated for 10 minutes before the monomer mixture was added. The surfactant, MA-80 forms a micellar domain; after modification the enzyme-pa-acrylic acid conjugate is cloudy (and slightly viscous) but goes clear

when added to MA-80 in water. Comonomers styrene-0.06% ($^{w}/_{v}$), and N-isopropylacrylamide (NiPAAm)-0.4 %,($^{w}/_{v}$), were added to the reaction with a cross-linker *N,N'*-Methylenebisacrylamide-0.53% ($^{w}/_{v}$) drop wise to the reactor and allowed to equilibrate for 10 minutes. A water soluble initiator, potassium persulfate (KPS)-0.16% ($^{w}/_{v}$) was added to initiate the polymerization. At this time, the temperature of the reaction was increased from 25°C to 40°C over a period of approximately 10 minutes.

Growing NiPAAm chains undergo a transition from being highly hydrophilic and swollen (at <32°C) to shrunken and hydrophobic (at temperatures above the LCST). As the reaction temperature is raised to 40°C, the NiPAAm becomes hydrophobic and the grafting reaction occurs. Once the reaction reaches 40°C (which takes approximately 10 minutes) the heat is removed and the stirred reactor is placed in an ice bath. A polymerization catalyst, tetramethylethylene (TEMED)-0.01% ($^{w}/_{v}$), is added to continue the free radical polymerization reaction while incubated on ice. The reaction is allowed to proceed for one hour after the addition of TEMED. After one hour, stirring is stopped and the round bottom flask is removed from the ice bath. After synthesis, samples were purified by equilibrium dialysis and centrifugation to remove unreacted monomer. After clean up the particles were tested for volume response to temperature, assayed for enzymatic activity, imaged and thoroughly studied for elevated temperature stability.

Task 6. Physical Characterization of Enzyme-Containing Particles

Dynamic light scattering (DLS) was used to determine the size distribution of particles in solution. A Malvern Zetasizer Nano ZS was used to determine nanoparticle size at various temperatures (as shown in Figure 2). The nanoparticles were diluted in filtered deionized water to a final concentration of 0.25% ($^{v}/_{v}$) before measurements were taken. DLS size readings were taken at 5 degree increments for GOx encapsulated nanoparticles from 25°C to 60°C. In their swollen state, the particles were approximately 95 nanometers (nm) and as temperature increased the particles shrunk by 50% to less than 50nm in diameter (as shown in Figure 2A). The size transition occurs at slightly above 40°C. OPH encapsulated nanoparticles were sized via DLS at one degree increments between 20°C and 60°C. The particles also shrunk by approximately 50% when the temperature increases above NiPAAm's LCST from approximately 85nm to 42nm (as shown in Figure 2B).

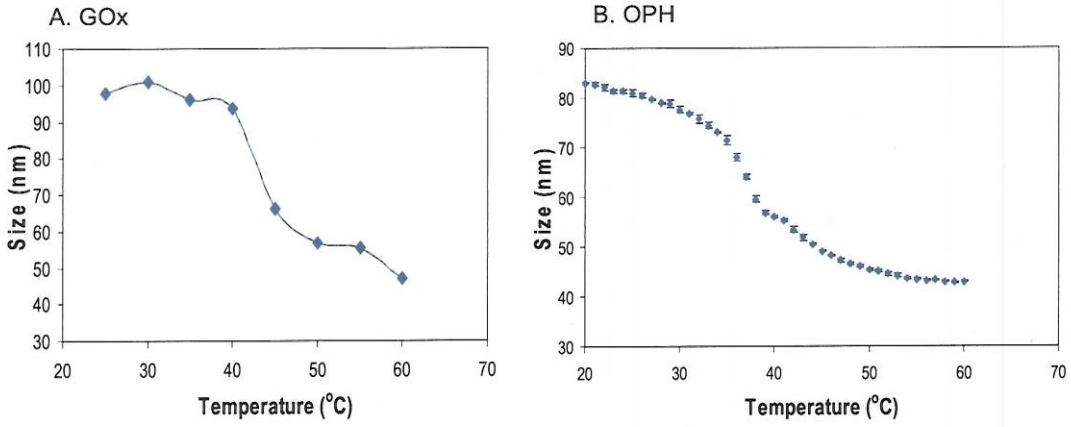

Figure 2. Particle volume transition, enzyme encapsulated NiPAAm particles undergo a ~50% volume transition when heated above NiPAAm's LCST

Standard assays were performed to monitor catalytic activity of enzymes. GOx activity is assayed at room temperature using a colorimetric assay coupled with horseradish peroxidase (HRP) in a buffered medium (100mM KPO_4, pH 7.5). GOx converts glucose to D-Glucono—lactone and H_2O_2. In the presence of H_2O_2, HRP converts 4-aminoantipyrine and N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine into quinoneimine dye, which is measured spectrophotometrically at 555nm.

OPH activity was assayed in a 96-well microplate using a buffered medium (10mM NaPO4, pH 7.0) supplemented with the pesticide paraoxon (5mM). OPH catalyzes the hydrolysis of paraoxon. OPH activity was assayed at room temperature by determining the increase of p-nitrophenol concentration in the presence of excess paraoxon. For each mole of paraoxon degraded, a mole of p-nitrophenol and a mole of phosphonic acid are produced. The rate of the reaction is directly proportional to the production of p-nitrophenol measured at 405nm.

Catalytic activity was measured throughout the modification and nanoparticle fabrication process to ensure that no step caused a significant loss in activity. An enzyme-friendly fabrication procedure was developed in order to minimize the loss of catalytic activity during the synthesis reaction.

Transmission electron microscopy (TEM) was utilized to determine particle size and relative monodispersity. As described above the particles were purified and a dilute dispersion of nanoparticles were plated on a carbon coated copper grid (Ted Pella, Inc, Redding, CA) and allowed to dry at ambient conditions. A Philips Mogagni 268 TEM was used to obtain images of thermo-responsive nanoparticles containing OPH. Figure 3 shows a 28k magnification of monodisperse particles that average 60nm in size (as shown in Figure 3. B).

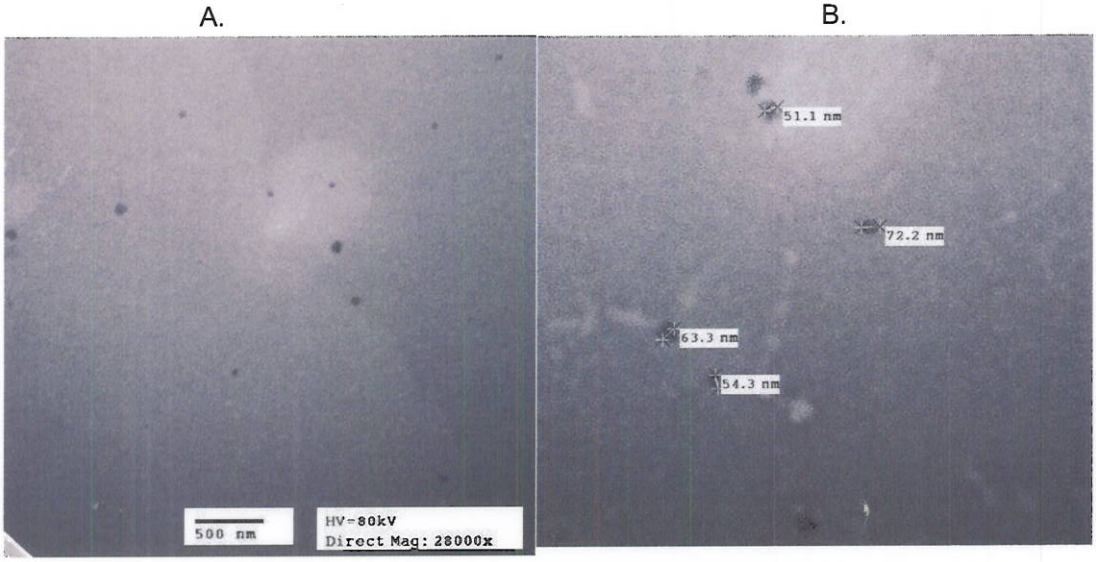

Figure 3. A Philips Mogagni 268 transmission electron microscope (TEM) was used at 28,000x magnification to obtain OPH encapsulated thermo-responsive nanoparticle images (A.). The zoomed in TEM image shows particles that average 60 nm in diameter (B.), which corresponds to our dynamic light scattering data.

Task 7. Core-shell Nanoparticle Response

During the past year, we have tested the response of thermally responsive nanoparticles to changes in temperatures to determine if encapsulation of enzymes into thermally responsive nanoparticles improves pot life due to contraction of the NiPAAm shell.

Aqueous solutions containing native GOx or OPH, functionalized GOx or OPH, as well as nanoencapsulated GOx or OPH were incubated at various temperatures and assayed to determine their thermal half-life (as shown in Table 1). Native and functionalized enzymes were added to nanoparticles, containing no enzyme, after particle synthesis to demonstrate that the adsorption of the enzyme or the presence of nanoparticles was not the cause of stability, but rather the encapsulation that increases thermal half-life. Non-enzyme containing nanoparticles were fabricated as previously described following a similar synthesis protocol. GOx samples containing 0.5mg/mL enzyme and 1mL nanoparticles or dH_2O and OPH samples containing 5mg/mL enzyme and 1mL nanoparticles or dH_2O were incubated at elevated temperatures and were assayed over the course of 24 hours to determine when each enzyme would reach its half-life of thermal inactivation.

Prior to incubation at elevated temperatures the catalytic activity was assayed; half-life of thermal inactivation is established when the samples lose 50% of their original activity. Encapsulated GOx samples incubated at 50°C increase their half-life by 8 times over unencapsulated controls; a 15-fold increase occurred at 60°C and at least a 2-fold increase in

A. GOx

Sample	50°C	60°C	70°C
GOx	2	<0.5	<0.25
GOx + Nanoparticles	2.5	<0.5	<0.25
Functionalized GOx	3.5	1	<0.5
Functionalized GOx + Nanoparticles	3.5	1	<0.5
Encapsulated GOx	**16**	**7.5**	**0.5**

B. OPH

Sample	50°C	60°C	70°C
OPH	2.5	1.5	1.5
OPH + Nanoparticles	3.5	1.5	1
Functionalized OPH	5.5	2	1.5
Functionalized OPH + Nanoparticles	6	2	2
Encapsulated OPH	**24**	**10**	**4**

Table 1. Half-life (hours) of thermal inactivation. The enzymatic activity in aqueous solutions was studied at elevated temperatures. GOx (A.) and OPH (B) samples were incubated at various temperatures and assayed periodically over the course of 24 hours.

thermal half-life occurs when incubated at 70°C (as shown in Table 1A). The same trends are seen with OPH and OPH encapsulated nanoparticles (as shown in Table 1B). Through encapsulation OPH samples increase their half life 10-fold at 50°C; a 6.67-fold increase in half-life is observed at 60°C; and the half-life of thermal inactivation is increased 2.67 times at 70°C. In all cases, enzymes encapsulated in NiPAAm nanoparticles retain a significantly higher

degree of catalytic activity at elevated temperatures and have drastically longer half-lives. Contraction of particles at elevated temperatures increases thermal pot life stability. It is hypothesized that the stability is diminished at 70℃ because the pNIPAAm is above its melting point and the stability is drastically reduced under these conditions. Solutions of encapsulated nanoparticles were observed to become quite viscous and gelatinous, indicating breakdown of the pNIPAAm at temperatures above the melting point.

Task 9. Assess Continuous Operation Lifetime of Particles

OPH encapsulated NiPAAm/Polystyrene nanoparticles were thermally cycled in a cuvette incubated in the Malvern Nano ZS. Particle size was measured every 5 degrees from 25°C to 60°C (as shown in Figure 4). The starting temperature of the cycles was alternated from 60°C to 25°C and then 25°C to 60°C for 6 cycles. Figure 4 A indicates that there is no significant hysteresis effect on the nanoparticles' size after 6 cycles. Nanoparticle diameter is consistent, at each temperature, from cycle to cycle. The particles continue to respond to temperature by undergoing a size change without breaking apart. The nanoparticles alongside native and functionalized OPH with and without non-enzyme containing nanoparticles were incubated in an Eppendorf mastercycler gradient thermal cycler. Non-enzyme containing particles were used as a control to establish that neither the presence of thermo-responsive nanoparticle nor enzyme adsorption onto the surface of the nanoparticle were responsible for artificially increasing enzymatic stability. Thermal cycles were set to mimic the DLS thermal stress study and after each thermal cycle, aliquots from each sample were removed and assayed for enzymatic activity. Encapsulated OPH retains its activity longer than functionalized or native OPH signifying that encapsulation protects OPH catalytic activity during repeated thermal cycling.

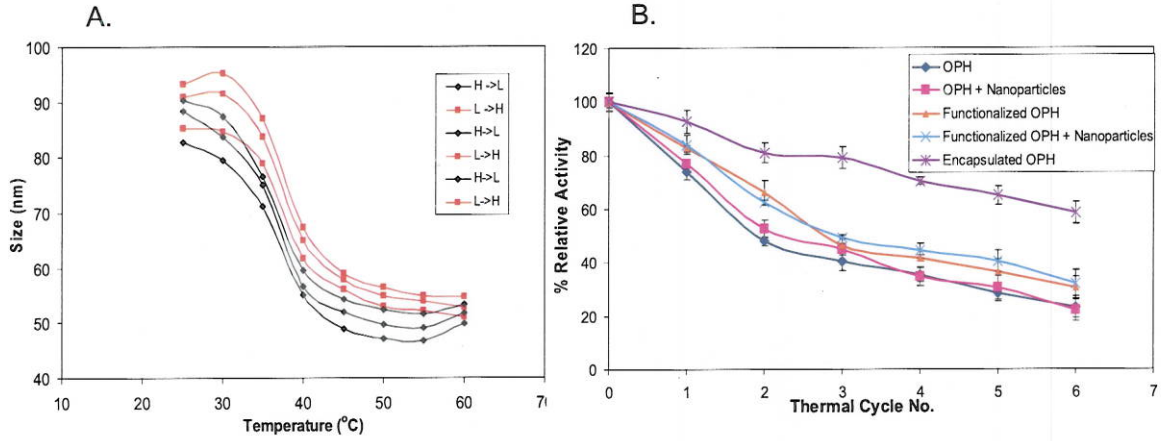

Figure 4. NiPAAm hysteresis: A. Particle NiPAAm encapsulated OPH particles undergo repeated thermal cycling between 25°C and 60°C. B. OPH samples were cycled between 25°C to 60°C to 25°C and assayed for catalytic activity.

Task 10. Determine Shelf Life of Particles

Lyophilization of enzymes is often used to extend shelf life while maintaining biological activity. The addition of stabilizer(s) assists in stabilizing the enzyme during the lyophilization process and further extends long term shelf life storage. Glucose oxidase encapsulated nanoparticles alongside native GOx was lyophilized without and with various amounts of stabilizer (data not shown) to determine the best ratio of stabilize to enzyme mass ratio. GOx and encapsulated GOx were lyophilized and thoroughly studied in the presence of a 50:1 mass ratio of a stabilizer, trehalose (as shown in Figure 5). After lyophilization the enzymes were assayed for catalytic activity and stored dry as aliquots at 70°C to determine shelf life. Lyophilization of these novel materials extends their shelf life over that of native enzyme. Encapsulated enzymes are catalytically active after storage at 70°C for over one and a half months; whereas native enzyme loses all activity by 3 weeks at 70°C (this work is currently ongoing). Shelf life of GOx at elevated temperatures is greatly enhanced by encapsulation into thermoresponsive nanoparticles

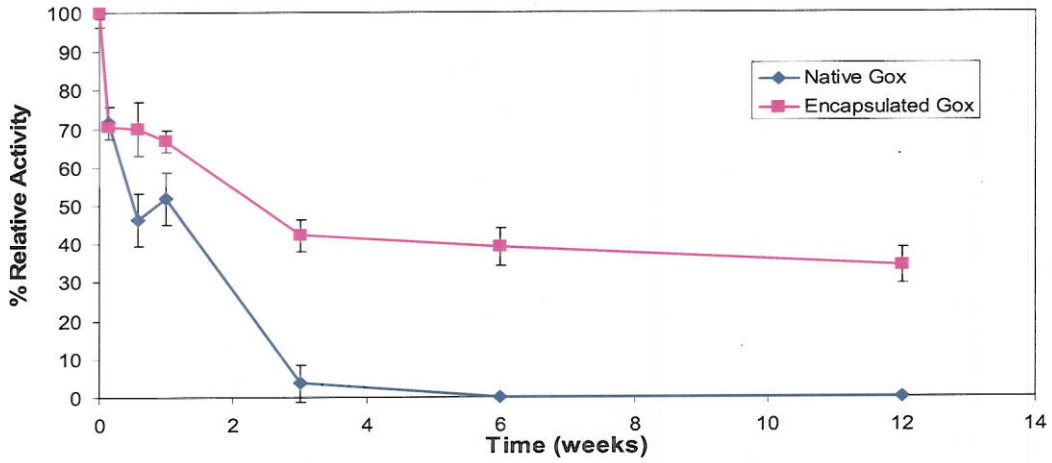

Figure 5. Shelf life stability at 70°C is greatly enhanced through encapsulation into thermoresponsive nanoparticles.

Future Work

In the upcoming year, ICx Agentase will focus its efforts on Task 8: Non-Specific Stimuli Testing of Particles as well as continuing our efforts on Task 10: Determine Shelf Life of Particles.

Task 8: Non-Specific Stimuli Testing of Particles

In the upcoming year, ICx Agentase will begin to evaluate enzyme encapsulated nanoparticles performance in real matrixes. We will subject them to a variety of chemical species including: solvents, acids, bases, gasoline, oil, oxidizers, other proteins, biological buffers and media, as well as real environmental samples. We will add the enzyme substrate to these interferent-containing samples and will perform the standard assays in the presence of the interferents to determine whether the particles retain their activity when the proper activating stimulus is

applied. We will also assess the effects that the non-specific stimuli have on the particle size by using the particle sizing instrument. This will allow us to understand whether the catalytic rate of the particles is slowed because the chemical species inactivates the enzyme or because it causes some change in the particle size which decreases the rate of diffusion of the substrate.

Task 10: Determine Shelf Life of Particles

In the upcoming year, ICx Agentase will continue its effort to evaluate the shelf life of enzyme encapsulated particles. Encapsulating GOx has increased its shelf life at 70°C from 3 weeks to over 3 months. Lyophilization and shelf life studies of OPH encapsulated nanoparticles will begin in the upcoming weeks. Initial data shows that the stabilizer, trehalose, used at a 50:1 mass ratio of stabilizer to enzyme, provides the most stability when performing accelerated aging tests. We will determine the actual shelf life of particles after the accelerated aging tests are complete by using the Arrhenius equation.

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